PCT/EP 00 / 05261 #2

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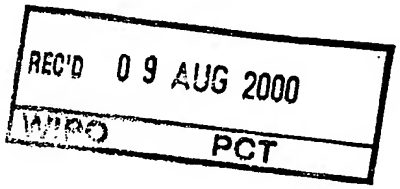


EP 00 5261 **Bescheinigung**

4

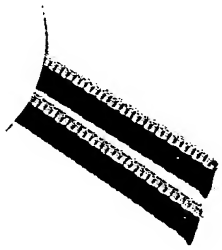
Die BASF Aktiengesellschaft in Ludwigshafen/Deutschland hat eine Patentanmeldung unter der Bezeichnung

"New calpains and use thereof (Neue Calpaine und deren Verwendung)"

am 18. Juni 1999 beim Deutschen Patent- und Markenamt eingereicht.

Die angehefteten Stücke sind eine richtige und genaue Wiedergabe der ursprünglichen Unterlagen dieser Patentanmeldung.

Die Anmeldung hat im Deutschen Patent- und Markenamt vorläufig die Symbole C 07 K, C 07 H und C 12 Q der Internationalen Patentklassifikation erhalten.



München, den 30. Mai 2000
Deutsches Patent- und Markenamt
Der Präsident
Im Auftrag

Dzierzon

Aktenzeichen: 199 28 021.5

A 9161
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11/98

Claims

1. Polypeptide having the amino acid sequence SEQID NO:2

2. Polynucleotide sequence coding for a polypeptide according to Claim 1

3. Polynucleotide sequence having the sequence SEQID NO:1

4. Use of a polypeptide according to Claim 1 for identifying agents which can inhibit the enzymatic activity of this polypeptide.

5. A method for identifying compounds which inhibit the enzymatic activity of a polypeptide according to Claim 1 comprising

   (a) comparing the amount of enzymatic activity of CAPN11 in the presence of the compound with the amount of enzymatic activity of CAPN11 in the absence of the compound and

   (b) selecting compounds which change the amount of enzymatic activity of CAPN11 compared to the enzymatic activity of CAPN11 in the absence of the compound.

6. Use of compounds identified by the method according to Claim 5 for treatment of male fertility disorders.

New calpains and use thereof

The invention relates to a new mammalian calpain, CAPN11, its synthesis and the use thereof.

Calpains are a superfamily of related proteins some of which have been shown to function as calcium-dependent cysteine proteases. In mammals, eight different calpains have been identified.

Calpains constitute a family of intracellular calcium-dependent cysteine proteases. An increasing number of mammalian calpain homologs have been identified and individual members can be classified into four classes based on physical structure and predicted properties. The class A 'classical calpains«, CAPN1, CAPN2, CAPN3 (p94), CAPN8 (nCL-2) and CAPN9 (nCL-4) are probably all protease-active and Ca2+-dependent. They consist of a variable large (80kDa) and an invariant small (30kDa) subunit. The class B and D calpains CAPN5 (6,15) and CAPN7 (8) are protease-active but most likely Ca2+-independent, the class C calpain CAPN6 probably possesses no protease activity. Calpains can also be categorized on the basis of their expression patterns with CAPN3, CAPN6, CAPN8 and CAPN9 exhibiting some tissue-specificity. The function of the calpains is unknown although they have been associated with a wide variety of physiological processes and pathological conditions (reviewed in Ref. 17). Identification of the entire spectrum of calpain family members is essential to elucidate their function and evolutionary history.

The invention relates to the new polypeptide CAPN11 having the amino acid sequence disclosed in SEQIDNO:2.

The new calpain CAPN11 protein possesses the features typical of calpains including potential protease and calcium-binding domains. It exhibits a highly restricted tissue distribution with the major site of expression being testis. Radiation hybrid mapping localized the gene to human chromosome 6, within a region mapped to p12. Phylogenetic analysis suggests that, in mammals, CAPN11 is most closely related to CAPN1 and CAPN2.

However, of the calpain sequences available, the predicted CAPN11 sequence exhibits greatest homology to the chicken μ/m calpain. Thus CAPN11 may be the human ortholog of μ/m calpain. The discovery of this new calpain emphasizes the complexity of the calpain family with members being distinguished on the basis of protease activity, calcium dependence and tissue expression.

The cDNA nucleotide sequence of the CAPN11 gene contains 2338 nucleotides (SEQ ID NO:1). The cDNA sequence was confirmed to be derived from a single mRNA by the successful amplification of the entire predicted coding region from human testis cDNA using flanking primers. Multiple cDNA clones were sequenced in their entirety to exclude any PCR-based artifacts.

One large open reading frame exists which encodes a protein of 702 amino acids (Mr of 80 kDa) (Fig. 1). The amino acid sequence shows similarity to the large subunit of members of the calpain family (Fig. 1). The protein can be subdivided into the four domains typical of calpain. Domain II shows the characteristics of a protease domain and the predicted amino acid sequence possesses the three amino acid residues (Cys102, His259 and Asn283) which are part of the active site of cysteine proteases (2). All five Ca2+ -binding sequences reported for CAPN2 (4,12) are conserved to some extent in the amino acid sequence (Fig. 1). Thus, this protein is likely to have protease and calcium-binding ability. A comparison of the predicted amino acid sequence with that of all other calpains revealed greatest sequence homology (57.5%) to chicken μ/m calpain. Of the mammalian calpains, the most similar was human CAPN1 (54.3% homology). The least similar human calpain, with only 18.7% homology, was CAPN6. The gene corresponding to this cDNA has been designated CAPN11 by the Human Gene Nomenclature Committee.

The complete amino acid sequences of all the identified human calpains were subjected to phylogenetic analysis. The results enable the human calpains to be classified into four major evolutionary groups (Fig. 2). The first group is represented by CAPN5, CAPN6, CAPN7 and CAPN8, the second by CAPN1 and CAPN2, the third group by CAPN3 and CAPN9 and the fourth contains CAPN11. Thus, the phylogenetic analysis suggests that CAPN11 represents a distinct calpain subfamily.

Expression of CAPN11 in human tissues was evaluated by Northern and RNA dot blot analysis. Of the 50 tissue RNAs tested, the CAPN11 mRNA was expressed at highest levels in testis (Fig. 3A). The specificity of this signal was verified by northern blot analysis and corresponded to an approximately 3Kb mRNA (Fig. 3D). Much weaker signals were detected in thymus and mammary gland but the significance of these is unclear as further investigation of thymus RNA using northern blot analysis did not reveal any signal despite long exposure times (Fig. 3D and data not shown). One possible explanation is that this weak signal is the result of cross-hybridization to related mRNAs. Nevertheless, testis is the major expression site of CAPN11 although we cannot rule out the

possibility that the gene is expressed in other tissues which were not tested.

We determined the chromosome location of the human CAPN11 gene. Using PCR with primers specific for the human CAPN11 nucleotide sequence the gene was mapped to chromosome six with a human/rodent somatic cell hybrid mapping panel (Correll Cell Repositories). With radiation hybrid mapping using the Stanford G3 medium resolution panel (Research Genetics Inc.) and the database at the Stanford Human Genome Center (shgc-www.stanford.edu) the gene was localized five centirays from the marker SHGC-32834 on this chromosome (LOD Score 12.87). This marker is located in the interval between the microsatellite markers D6S1616 (59.6 cM) and D6S427 (73.9 cM) (7) and a marker in this interval, D6S269, has been mapped cytogenetically to 6p12 (5). Thus CAPN11 is located on chromosome 6 in the vicinity of p12. No other calpain gene has been located on this chromosome.

The chicken μ/m calpain was the first member of the calpain family to be cloned (16). It was originally designated as m-calpain but reclassified after the identification of other chicken calpains most likely orthologous to the mammalian μ- and m-calpains (18). A mammalian μ/m calpain has remained elusive but as CAPN11 shows greater homology to chicken μ/m calpain than to any of the mammalian calpains it may be its ortholog.

There are now five calpains which exhibit some degree of tissue-specificity - CAPN3 (skeletal muscle), CAPN6 (placenta), CAPN8 (possibly smooth muscle), CAPN9 (stomach and small intestine) and CAPN11 (testis). Numerous proteases have been identified in testis and have been proposed to participate in processes such as tissue reorganization (20), regulation of spermatogenesis (14), penetration of the zona pellucida by sperm (10) and fertility (13). Many of these activities are, however, dependent on secreted proteases and CAPN11 is likely to be localized intracellularly. In testis it could participate in processes with which calpains have been associated in other tissues such as germ cell apoptosis (3) or regulation of testis-specific transcription factors.

Another aspect of this invention is the use of the polypeptide CAPN11 for identifying agents which can inhibit the enzymatic activity of this polypeptide, so called calpain-inhibitors, especially those calpain inhibitors which are selecetive for CAPN11. Selectivity means that those calpain inhibitors inhibit

the activity of CAPN11 stronger than the activity of other calpains mentioned above, preferably at least 10 times, more preferred 25 times stronger.
The enzymatic activity of CAPN11 is a Ca-dependent protease activity.

Another aspect of the invention is a method for identifying compounds which inhibit enzymatic activity of a polypeptide according to claim 1 comprising

(a) comparing the amount of enzymatic activity of CAPN11 in the presence of the compound with the amount of enzymatic activity of CAPN11 in the absence of the compound and

(b) selecting compounds which change the amount of enzymatic activity of CAPN11 compared to the enzymatic activity of CAPN11 in the absence of the compound.

The inhibiting compounds identified by the method mentioned above are useful for the treatment of diseases which are associated or linked with an unphysiologically elevated activity of CAPN11 such as male infertility.

The dosage and the treatment regimen of those inhibitors have to be determined by routine procedures which are well known from other protease inhibitors.

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Figures

FIG. 1. Alignment of the predicted amino acid sequence of CAPN11 to other human calpains. Multiple alignment of amino acid sequences was carried out using CLUSTAL W (19). The proposed initiation methionine for CAPN11 (GGAatgG) conforms to the minimal consensus sequence for the translational start site (RNNatgG, where R is a purine; Ref. 11). Amino acids identical in the other proteins with those of CAPN11 shaded. Dashes indicate gaps introduced to maximize alignment. Arrow heads indicate the three conserved amino acids which are part of the active site of calpains. The potential EF-hand calcium-binding domains of CAPN8 are underlined and numbered sequentially. The arbitrary domains of calpain are indicated. Not shown are the sequences for CAPN4 and CAPN7 which have only been identified in rat and mouse respectively. The published CAPN6 sequence was not included as it is only partial. The alternative names and accession numbers for the calpains aligned are given in the legend to Fig. 2.

FIG. 2. Unrooted phylogenetic tree of the human calpain large subunit family. Analysis was performed using the PAUP program and the tree assembled with CLUSTREE from the HUSAR server at the German Cancer Research Center, Heidelberg (www.dkfz-heidelberg.de). Lengths of horizontal lines are proportional to the inferred phylogenetic distances; vertical lines have no significance. 1000 bootstrap repetitions were performed and values are shown at the inner nodes. The Capn7 sequence is that of the mouse as little human nucleotide and protein sequence is available. Nevertheless the human ortholog does exist (see Ref. 8) justifying the use of the mouse sequence for this comparison. The partial human CAPN8 sequence is the predicted translation from the EST clone AA026030 (Hillier et al., 1995, The WashU-Merck EST Project, unpublished results). An amino acid translation of

this clone exhibits high similarity to the rat Capn8 sequence. No bootstrapping was performed with this sequence as it is much shorter than the others and thus the bootstrap value can not be meaningfully compared to the values derived from full-length sequence comparisons. The nomenclature specified by the Human Gene Nomenclature Committee is used. Previous names for the various calpains are: CAPN1 - m-calpain; CAPN2 - m-calpain; CAPN3 - p94, nCL-1; CAPN8, nCL-2; CAPN9, nCL-4. The EMBL accession numbers for the calpain sequences used are CAPN1 (P17655), CAPN2 (P07384), CAPN3 (P20807), CAPN5 (Y10656), CAPN6 (Y12582), Capn7 (AJ012475) and CAPN9 (AF022799).

FIG. 3. Expression of CAPN11. A 32P-labeled DNA probe containing an 800 base-pair segment of the human CAPN11 cDNA coding sequence was hybridized to a Master Blot (A), a nylon filter containing dot blots of RNAs from 50 different human tissues, or a Clontech Multiple Tissue Northern Blot (D). Filters were washed at high stringency (6xSSC, 65;C). The precise location of the various RNAs on the dot blot filter is shown schematically (C). RNAs on the northern blot are indicated above the relevant lanes. The dot blot and northern blot were rehybridized with human ubiquitin (B) and b-actin DNA probes respectively to confirm amounts of poly(A+) RNA loaded. For the northern blot, the position of the size markers (in kilobases) are indicated. The exposure times were: A, 72 hours; B, 24 hours; D, 48 hours. PBL = peripheral blood leukocytes.

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Pro Ala Glu Pro Ser Ser Leu Gly Phe Lys Asp Leu Gly Pro Asn Ser
     50                  55                  60

Lys Asn Val Gln Asn Ile Ser Trp Gln Arg Pro Lys Asp Ile Ile Asn
 65                  70                  75                  80
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Asn Pro Leu Phe Ile Met Asp Gly Ile Ser Pro Thr Asp Ile Cys Gln
                  85                  90                  95

Gly Ile Leu Gly Asp Cys Trp Leu Leu Ala Ala Ile Gly Ser Leu Thr
             100                 105                 110

Thr Cys Pro Lys Leu Leu Tyr Arg Val Val Pro Arg Gly Gln Ser Phe
         115                 120                 125

Lys Lys Asn Tyr Ala Gly Ile Phe His Phe Gln Ile Trp Gln Phe Gly
     130                 135                 140

Gln Trp Val Asn Val Val Val Asp Asp Arg Leu Pro Thr Lys Asn Asp
145                 150                 155                 160

Lys Leu Val Phe Val His Ser Thr Glu Arg Ser Glu Phe Trp Ser Ala
                165                 170                 175

Leu Leu Glu Lys Ala Tyr Ala Lys Leu Ser Gly Ser Tyr Glu Ala Leu
             180                 185                 190

Ser Gly Gly Ser Thr Met Glu Gly Leu Glu Asp Phe Thr Gly Gly Val
         195                 200                 205

Ala Gln Ser Phe Gln Leu Gln Arg Pro Pro Gln Asn Leu Leu Arg Leu
     210                 215                 220

Leu Arg Lys Ala Val Glu Arg Ser Ser Leu Met Gly Cys Ser Ile Glu
225                 230                 235                 240

Val Thr Ser Asp Ser Glu Leu Glu Ser Met Thr Asp Lys Met Leu Val
                245                 250                 255

Arg Gly His Ala Tyr Ser Val Thr Gly Leu Gln Asp Val His Tyr Arg
             260                 265                 270

Gly Lys Met Glu Thr Leu Ile Arg Val Arg Asn Pro Trp Gly Arg Ile
         275                 280                 285

Glu Trp Asn Gly Ala Trp Ser Asp Ser Ala Arg Glu Trp Glu Glu Val
     290                 295                 300

Ala Ser Asp Ile Gln Met Gln Leu Leu His Lys Thr Glu Asp Gly Glu
305                 310                 315                 320

Phe Trp Met Ser Tyr Gln Asp Phe Leu Asn Asn Phe Thr Leu Leu Glu
                325                 330                 335

Ile Cys Asn Leu Thr Pro Asp Thr Leu Ser Gly Asp Tyr Lys Ser Tyr
             340                 345                 350

Trp His Thr Thr Phe Tyr Glu Gly Ser Trp Arg Arg Gly Ser Ser Ala
         355                 360                 365

Gly Gly Cys Arg Asn His Pro Gly Thr Phe Trp Thr Asn Pro Gln Phe
     370                 375                 380

Lys Ile Ser Leu Pro Glu Gly Asp Asp Pro Glu Asp Asp Ala Glu Gly
385                 390                 395                 400
```

Asn Val Val Val Cys Thr Cys Leu Val Ala Leu Met Gln Lys Asn Trp
405 410 415

Arg His Ala Arg Gln Gln Gly Ala Gln Leu Gln Thr Ile Gly Phe Val
420 425 430

Leu Tyr Ala Val Pro Lys Glu Phe Gln Asn Ile Gln Asp Val His Leu
435 440 445

Lys Lys Glu Phe Phe Thr Lys Tyr Gln Asp His Gly Phe Ser Glu Ile
450 455 460

Phe Thr Asn Ser Arg Glu Val Ser Ser Gln Leu Arg Leu Pro Pro Gly
465 470 475 480

Glu Tyr Ile Ile Ile Pro Ser Thr Phe Glu Pro His Arg Asp Ala Asp
485 490 495

Phe Leu Leu Arg Val Phe Thr Glu Lys His Ser Glu Ser Trp Glu Leu
500 505 510

Asp Glu Val Asn Tyr Ala Glu Gln Leu Gln Glu Glu Lys Val Ser Glu
515 520 525

Asp Asp Met Asp Gln Asp Phe Leu His Leu Phe Lys Ile Val Ala Gly
530 535 540

Glu Gly Lys Glu Ile Gly Val Tyr Glu Leu Gln Arg Leu Leu Asn Arg
545 550 555 560

Met Ala Ile Lys Phe Lys Ser Phe Lys Thr Lys Gly Phe Gly Leu Asp
565 570 575

Ala Cys Arg Cys Met Ile Asn Leu Met Asp Lys Asp Gly Ser Gly Lys
580 585 590

Leu Gly Leu Leu Glu Phe Lys Ile Leu Trp Lys Lys Leu Lys Lys Trp
595 600 605

Met Asp Ile Phe Arg Glu Cys Asp Gln Asp His Ser Gly Thr Leu Asn
610 615 620

Ser Tyr Glu Met Arg Leu Val Ile Glu Lys Ala Gly Ile Lys Leu Asn
625 630 635 640

Asn Lys Val Met Gln Val Leu Val Ala Arg Tyr Ala Asp Asp Asp Leu
645 650 655

Ile Ile Asp Phe Asp Ser Phe Ile Ser Cys Phe Leu Arg Leu Lys Thr
660 665 670

Met Phe Thr Phe Phe Leu Thr Met Asp Pro Lys Asn Thr Gly His Ile
675 680 685

Cys Leu Ser Leu Glu Gln Trp Leu Gln Met Thr Met Trp Gly
690 695 700

Abstract

New calpains and use thereof

Fig. 1

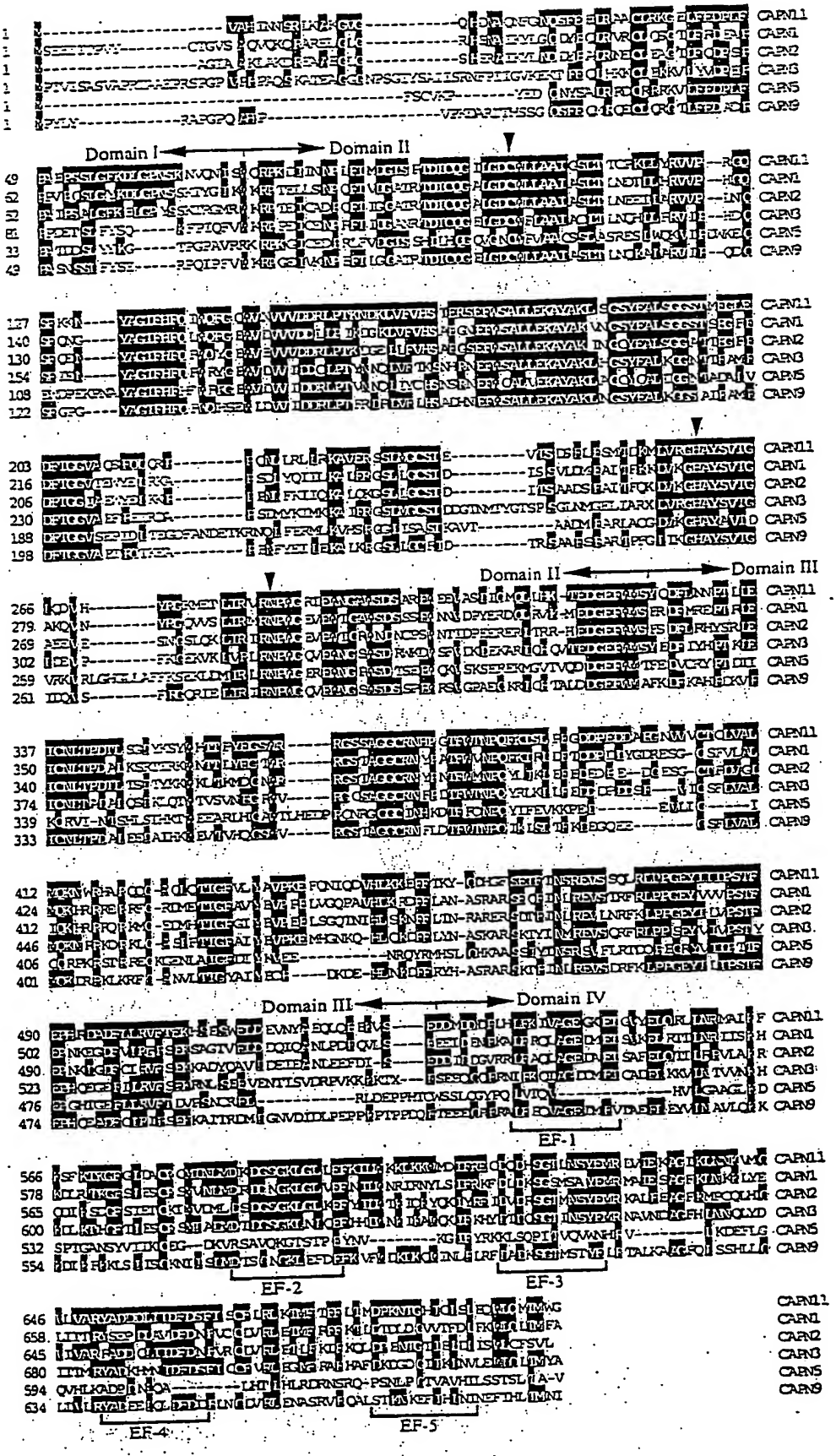
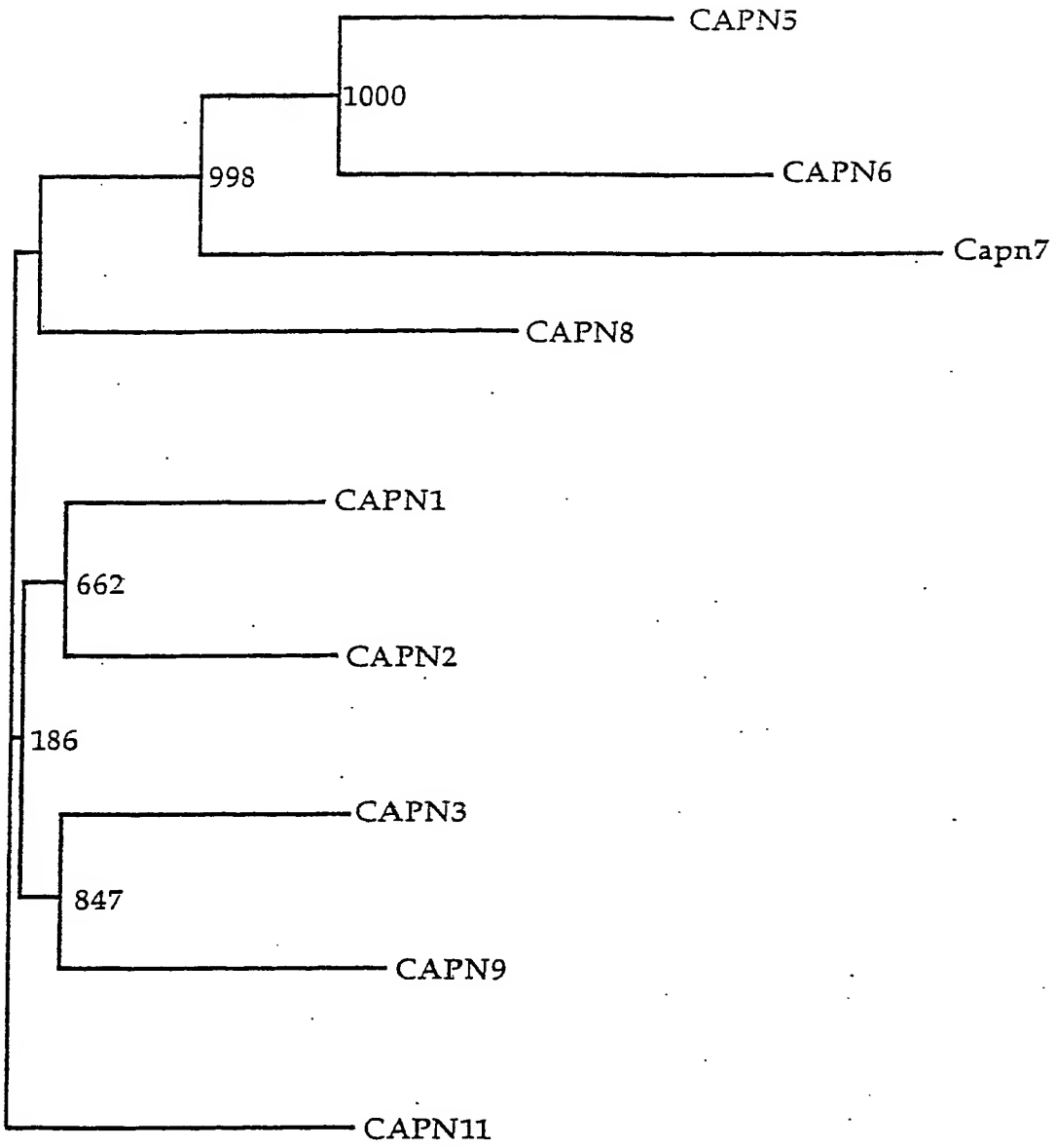

Domain I
Domain II
Domain II
Domain III
Domain III
Domain IV
EF-1
EF-2
EF-3
EF-4
EF-5
CAPN11
CAPN1
CAPN2
CAPN3
CAPN5
CAPN9

Fig. 2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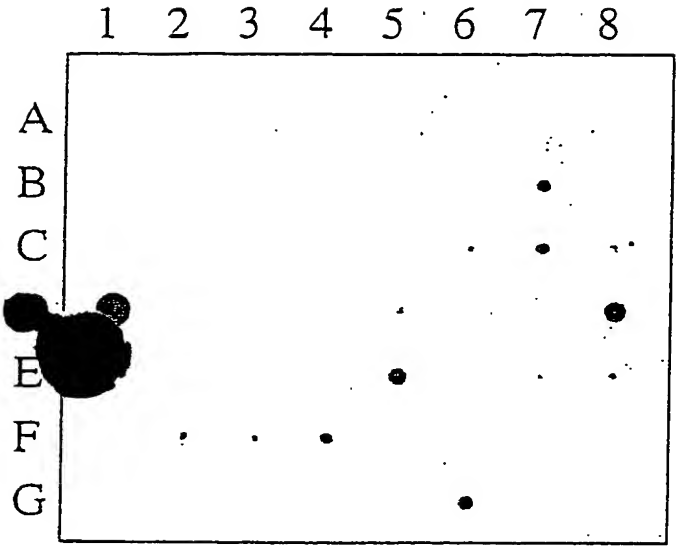
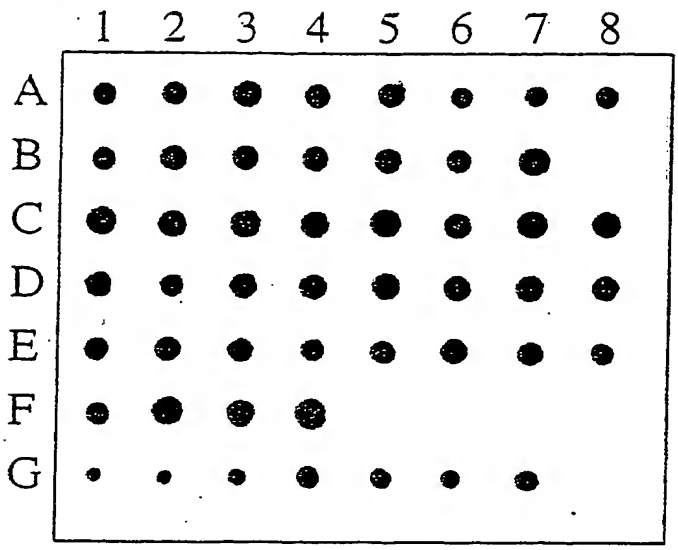
1000
CAPN6
998
Capn7
CAPN8
CAPN1
662
CAPN2
186
CAPN3
847
CAPN9
CAPN11

Fig. 3

A.



B.



C.

	1	2	3	4	5	6	7	8
A	whole brain	amygdala	caudate nucleus	cere-bellum	cerebral cortex	frontal lobe	hippo-campus	medulla oblongata
B	occipital lobe	putamen	substantia nigra	temporal lobe	thalamus	nucleus accumbens	spinal cord	
C	heart	aorta	skeletal muscle	colon	bladder	uterus	prostate	stomach
D	[illegible]	ovary	pancreas	pituitary gland	adrenal gland	thyroid gland	salivary gland	mammary gland
E	kidney	liver	small intestine	spleen	thymus	peripheral leukocyte	lymph node	bone marrow
F	appendix	lung	trachea	placenta				
G	fetal brain	fetal heart	fetal kidney	fetal liver	fetal spleen	fetal thymus	fetal lung	

D.